

Thymus Immunoglobulin Receptors

ONTOGENIC DEVELOPMENT, RESPONSE TO ANTIGENIC STIMULATION AND THEIR POSSIBLE ROLE IN THE REGULATION OF 'AGGRESSIVE' IMMUNE REACTIONS

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Summary. IgG, IgA and IgM receptors were detected on mouse thymocytes with a cytotoxic assay. IgG receptors were only detectable during the postnatal period, while the IgA and IgM were also present on thymocytes from adults. IgA cells were more abundant in neonates. Thymus receptors of the three classes were immunochemically unrelated to spleen receptors. IgA and IgM thymus cells responded to antigenic stimulation with SRBC by proliferation and exposure of new, class-specific antigenic determinants, whereas spleen cells did not. The possible role of thymus receptors may be regulatory by inhibition of the humoral and cellular 'aggressive' immune reactions.

INTRODUCTION

Surface immunoglobulins probably play an important role as receptors for recognition and binding of antigens. Their presence on B cells in the mouse is well documented (Raff, 1970; Rabellino, Colon, Grey and Unanue, 1971; Unanue, Grey, Rabellino, Campbell and Schmidtke, 1971; Takahashi, Old, McIntire and Boyse, 1971; Baur, Vitetta, Sherr, Schenkein and Uhr, 1971; Vitetta, Baur and Uhr, 1971; Vitetta, Bianco, Nussenzweig and Uhr, 1972; Vitetta and Uhr, 1972a, b; Perkins, Karnovsky and Unanue, 1972; Lamelin, Lisowska-Bernstein, Matter, Ryser and Vassalli, 1972) but only some workers have reported finding them on T cells (Nossal, Warner, Lewis and Sprent, 1972) and on normal thymocytes (Nossal *et al.*, 1972; Grey, Colon, Campbell and Rabellino, 1972; Marchalonis, Atwell and Cone, 1972; Marchalonis, Cone and Atwell, 1972; Cone, Sprent and Marchalonis, 1972).

Recently we isolated and characterized a new specific differentiation antigen, the GL-antigen, which has a distribution similar to B cells in the adult mouse (Fellenberg and Guggisberg, 1972). The function of this surface protein is unknown, so we have studied its ontogenic development, hoping to be able to correlate its presence with the development of known immune functions. In the spleen the relative number of cells expressing the antigen increases steadily and reaches adult values at 4 weeks of age.

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In the thymus however, a relatively large number of cells was found only during the first 2 weeks of life, with a maximum of 44 per cent on the 5th day.

This unexpected result stimulated us to look for surface immunoglobulin receptors in the thymus, first during the postnatal period and later in adult animals. For this purpose we used class-specific antisera to IgA, IgM and IgG and a cytotoxic assay (Gorer and O'Gorman, 1956; Takahashi *et al.*, 1971; Miller, Sprent, Basten and Warner, 1972; Wernet, Feizi and Kunkel, 1972). These results were as unexpected as those with the GL antigen. They showed that α - and γ -chain receptor cell pools expanded and regressed during the 1st week of life in the thymus. The same phenomenon was observed with α -receptor cells in the spleen. In contrast, μ -receptor cells developed continuously in the thymus and the spleen. The thymus receptors of all three classes did not cross-react immunochemically with spleen cell receptors. In the adult, the α - and μ -thymus receptor cells responded selectively to short time immunization with sheep red blood cells (SRBC) by proliferation and exposure of new, class-specific antigenic determinants.

At the present time the biological meaning of our results remains obscure. We have attempted a critical discussion and have formulated a hypothesis based on the bridging theory (Mitchison, Rajewski and Taylor, 1970; Raff, 1973) that the thymus receptors could be responsible for tolerance and self recognition in the neonate and regulation of the 'aggressive' immune reactions in the adult.

MATERIALS AND METHODS

All chemicals used were analytical grade commercial products. Mouse Cohn fraction II (lot 16) was obtained from Pentex Inc., Kankakee, Illinois, and commercial antisera against mouse serum from goat (61-71) and rabbit (4-014) from Miles-Seravac, England. Rabbit antisera to IgM myeloma protein (MOPC 104 E) and to IgA myeloma protein (MOPC 315) were a gift from Dr Jaquet, Institut de Biochemie, Lausanne, Switzerland.

Animals

Swiss mice from a closed colony and inbred C57/B16 mice were used. New Zealand white rabbits and guinea-pigs were used for production of the antisera and as a source of complement.

Preparative methods

Purification of Cohn fraction II. The γ -globulin used for the induction of anti-IgG serum was obtained by chromatography of Cohn fraction II on DEAE-cellulose equilibrated with 0.005 M sodium phosphate of pH 8.0.

Isolation of L-chains. Free L-chains were obtained by the method of Williamson and Askonas (1968) from Cohn fraction II.

Purification of a fraction from mouse serum consisting of IgM and IgA. The fraction was purified from the serum of mice which had been injected 4 days previously with SRBC and was used as immunogen for induction of anti-IgM and anti-IgA. The procedures of two published methods were combined (Chesebro, Bloth and Svehag, 1968; Van Breda, Vriesman and Feldman, 1972). Aliquots of 25 ml of serum were dialysed against 0.06 M sodium phosphate buffered at pH 7.6 and passed through a DEAE-cellulose column equilibrated with the same buffer. The column was rinsed with buffer and the protein not absorbed was discarded. The retained macroglobulins were eluted with 1.0 M NaCl in



phosphate buffer and dialysed against 0.15 M NaCl. One volume of a mixture of 1 per cent dextran sulfate and 0.18 M CaCl_2 was added to the protein solution. One hour later the precipitated lipoproteins were sedimented for 30 minutes at 15,000 *g* and discarded. Polyethylene glycol was added to the supernatant to give a final concentration of 7.5 per cent (w/v). The macroglobulins precipitated during 24 hours incubation in the cold were then centrifuged for 60 minutes at 15,000 *g*. The sediment was dissolved in 1.0 ml of a mixture of 0.5 M NaCl and 2 per cent butanol buffered with 0.05 M sodium phosphate at pH 7.2 and dialysed against the same solvent. A precipitate formed and was centrifuged off. The protein solution was fractionated on a Sephadex G-200 column (1.6 $\times$ 92 cm) equilibrated with the same solvent. The fractions of the void volume were pooled and dialysed over night against 0.06 M sodium phosphate (pH 7.6). This IgM/IgA fraction was concentrated by absorption on and elution from a short (2.0 $\times$ 4.5 cm) DEAE-cellulose column as already described. The recovery was about 0.3 mg protein from 25 ml serum. The final products of five preparations were pooled and again concentrated on DEAE-cellulose to about 1 mg protein/ml.

Electrophoretic preparation of IgA. 0.3 ml of the IgM/IgA preparation was submitted to micropreparative electrophoresis on microscope slides. The system was buffered in the same way as for immunoelectrophoresis but agarose instead of agar was used as supporting medium. After a running time of 120 minutes IgM remained near the origin but IgA localized in an anodic position. The area containing only IgA was cut out, extracted with buffer and injected into rabbit 1904 as a boost for anti-IgA production.

Gel filtration of serum. Sephadex G-200: A 1.5 $\times$ 75 cm column equilibrated with phosphate-buffered saline (pH 7.2) was used. 1.0 ml samples were fractionated and collected in fractions of 2.0 ml. All anti-Ig sera were separated on this column since only the 7S antibody fraction was used for the cytotoxic assay. This step removed soluble immune complexes after absorptions. Serum of SRBC-stimulated mice was also fractionated on the column. The crude IgM fraction in the void volume was used to absorb anti- μ -chain serum completely for control experiments. Sepharose 6B: A 3.0 $\times$ 56 cm column equilibrated with phosphate-buffered saline of pH 7.2 was used. Five millilitres serum of SRBC-stimulated mice was fractionated. An IgM-rich fraction in the void volume was separated from an IgA-rich fraction which was retained. The elution profile is represented in Fig. 1. Both fractions were used for absorption of anti-IgM and anti-IgA sera.

Analytical methods

Cytotoxic assay (Gorer and O'Gorman, 1956; Takahashi *et al.*, 1971; Miller *et al.*, 1972; Wernet *et al.*, 1972).

(1) *Cell suspensions.* The cells from six to eight young adult donors were suspended in Hanks' balanced salt solution containing 0.1 per cent BSA, filtered through glass wool and washed three times. They were then resuspended in the buffer used for the assay (see below) and adjusted to a concentration of 25×10^6 cells per ml. The cells from whole litters (ten to fifteen individuals) were used for the experiments on ontogenetic development.

(2) *Assay.* Tris-buffered saline of pH 7.2 containing 0.1 per cent BSA and optimal Ca^{++} and Mg^{++} concentrations was used as diluent (Levine, 1967). Starting with a 1:10 dilution six to twelve serial two-fold antiserum dilutions were prepared for each test

according to the potency of the antiserum. 0.2 ml diluted antiserum was added to 0.2 ml cell suspension (5×10^6 cells), followed by 0.2 ml C diluted 1:2. All tubes were kept in an ice bath. For the assay they were incubated for 30 minutes at 37° . The reaction was stopped by putting the tubes in the ice bath again and by addition of 0.4 ml ice-cold diluent. 100 cells from each tube were counted twice within 2 minutes after addition of Trypan Blue on a microscope slide. Controls included substitution of normal sera for the antisera; diluent for the antisera; diluent for C. The dead cell values of these controls were 4 per cent to 7 per cent and were deducted from the dead cells of the assay.

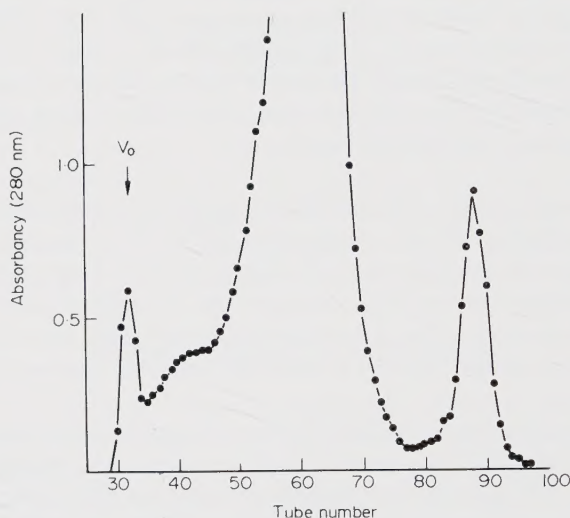


FIG. 1. Sepharose 6B gel-filtration chromatography on a 3.0×56 cm column. Fractionation of 5.0 ml mouse serum obtained 4 days after immunization with SRBC. The serum was separated into an IgM-rich fraction in the void volume (V_0) and an IgA-rich fraction collected in tubes 40–50. These fractions were used for absorption of anti- μ - and anti- α -chain sera.

Complement source. Rabbit C was used with the rabbit antisera and guinea-pig C with the guinea-pig antisera. C from both species was absorbed on agarose (Cohen and Schlesinger, 1970). In addition, rabbit C was absorbed with SRBC ghosts.

Haemolytic plaque assay. The microscope slide modification of the direct plaque assay was used (Mishell and Dutton, 1967).

Immuno-electrophoresis. Immuno-electrophoresis was performed on microscope slides in 2 per cent agar and 0.05 M barbital buffer of pH 8.6. A current of 6 mA per slide (60 V) was applied for 80 minutes.

Double gel diffusion. Double gel diffusion was performed in barbital-buffered agar. The wells had a diameter of 5 mm and contained 70–80 microlitres. The distance between the wells was 4–5 mm.

Indirect immunofluorescence staining of living thymus cells. The conditions have been described elsewhere (von Fellenberg, Briner and Guggisberg, 1971, von Fellenberg *et al.*, 1972). Controls consisted of anti-IgG serum which had been absorbed completely with Cohn fraction II.

Antisera

Rabbits were injected in the footpads with 0.5 ml protein solution emulsified in an equal volume of Freund's complete adjuvant. Three weeks later the animals were boosted by the same route. Five weeks after the initial injection the animals were bled. Guinea-pigs were immunized according to the same schedule, but were injected subcutaneously with a final volume of 0.3 ml of emulsion.

Anti-IgG sera. Rabbit 2784 was immunized with 150 μ g γ -globulin per injection. Rabbit 17 was inoculated with 35 μ g protein per injection.

Anti-IgM sera. Rabbit 26 was immunized with two injections of 65 μ g of the IgM/IgA-containing preparation and rabbit 44 with two injections of 150 μ g of the same antigen. The guinea-pigs 48 and 49 were immunized with two injections of 50 μ g of the IgM/IgA preparation.

TABLE 1
THE EFFECT OF ABSORPTION WITH RED CELLS ON THE NON-SPECIFIC
CYTOTOXICITY FOR LYMPHOID CELLS OF RABBIT ANTISERA

Serum	Red cells used for absorption	Percentage dead target cells	
		Spleen	Thymus
Anti-ovalbumin 2768, 1/10	None	17	—
	Sheep	12	—
	Sheep+mouse	0	0
Anti-ovalbumin 2791, 1/10	None	25	—
	Sheep	15	—
	Sheep+mouse	0	0
Anti-RNase 1941, 1/10	None	25	—
	Sheep	13	—
	Sheep+mouse	0	0
NRS 1/10	None	2	0
NRS 1/10	None	0	0
NRS 1/10	None	0	0

— = not done.

0 = no detectable cytotoxic reaction.

Anti-IgA serum. Rabbit 1904 was injected twice with 100 μ g of the IgM/IgA preparation. It was rested for 5 months and then boosted with 100 μ g IgA which had been purified electrophoretically. Two weeks after the last injection the rabbit was bled.

Absorption of the anti-Ig sera to remove non-specific cytotoxic antibodies. Antisera from rabbits receiving Freund's complete adjuvant contained non-specific cytotoxic antibodies which could be removed by absorption with sheep and mouse erythrocytes. This is demonstrated in Table 1 with three rabbit antisera to non-related antigens. The heat-inactivated anti-Ig sera were absorbed in the ratio of 4 ml antiserum to 1.0 ml packed SRBC and to 0.2 ml packed mouse erythrocyte ghosts. The guinea-pig antisera were further treated with agarose (40 mg/ml serum) to remove naturally occurring anti-thymocyte antibodies (Cohen *et al.*, 1970). Antiserum portions which were not further absorbed were centrifuged for 100 minutes at 105,000 *g*, filtered through the Sephadex G-200 column and the 7S antibody fractions were concentrated to the original volume.

Class-specific antisera. Anti- γ -chain serum. Rabbits 2784 and 17 were free of contaminant antibodies to serum proteins other than γ -globulin as judged by immunoelectrophoresis

with mouse serum and Cohn fraction II as antigens. Serum from rabbit 2784 showed a weak reaction in double gel diffusion with the purified IgM/IgA preparation indicating that the antiserum contained a low anti-L chain activity. One millilitre of antiserum was rendered specific for γ -chains by absorption with 0.7 mg of isolated L-chains. The antiserum was then ultracentrifuged, filtered through Sephadex G-200 and the 7S peak concentrated as described above.

Anti- μ -chain sera. One millilitre-aliquots of the antisera (rabbits 26 and 44, guinea-pigs 48 and 49) were first absorbed with 8–13 mg serum protein from 2–5-day-old mice, followed by 1.0–4.0 mg protein of the IgA-rich fraction of mouse serum after Sepharose 6B separation. Ultracentrifugation, filtration through Sephadex G-200 and reconcentration of the 7S fraction followed these absorptions. The μ -chain specificity was ascertained by immunoelectrophoresis and double gel diffusion using the antiserum to monoclonal IgM (MOPC 104 E) as a reference and serum from SRBC-stimulated mice as the antigen (Fig. 2).

Anti- α -chain serum. The antiserum (rabbit 1904) was first absorbed with neonatal mouse serum as described above for the anti- μ -chain sera. Then it was absorbed with 0.5 mg protein from the IgM-rich fraction (exclusion volume) of mouse serum after separation on Sepharose 6B. Ultracentrifugation, fractionation on Sephadex G-200 and reconcentration to the original volume followed absorption of the antiserum. The specificity was verified by immunoelectrophoresis and double gel diffusion using antiserum to monoclonal IgA (MOPC 315) as a reference (Fig. 2b).

Complete absorption of the class-specific antisera for control experiments. Anti-IgG serum 2784, 1.0 ml, was absorbed with 10 mg of Cohn fraction II. Anti- μ -chain serum 26, 1.0 ml, was absorbed with 16 mg protein of the IgM-rich fraction (void volume) of SRBC-stimulated mouse serum after separation on Sephadex G-200. Anti- α -chain serum 1904, 1.0 ml, was absorbed with 20 mg protein of the IgA-rich fraction of mouse serum after separation on Sepharose 6B. Again the antisera were ultracentrifuged, fractionated on Sephadex G-200 and reconcentrated to the original volume. Complete absorption of the three antiserum portions was confirmed in double gel diffusion.

Absorption of the antisera with crude membranes of spleen, thymus and brain. Crude membranes were prepared according to a previously published method (von Fellenberg, 1971). Twelve milligrams of membrane protein was the unit amount to absorb 1.0 ml of an antiserum. After absorption overnight in the cold the antisera were centrifuged for 100 minutes at 105,000 g.

RESULTS

SURFACE RECEPTORS REACTING WITH ANTI-IGG SERUM

Postnatal development of receptor cells in thymus and spleen

Thymus and spleen cells were assayed daily after birth for surface receptors reacting with anti-IgG serum. The results are represented in Fig. 3. At birth 2 per cent of the thymocytes reacted with anti-IgG serum in the cytotoxic assay. The relative amount then increased sharply until day 5 and after 7 decreased rapidly. The adult value of 2 per cent was reached on day 22. The development in the spleen was different. At birth no receptor cells were detectable. They appeared on day 4 and increased continuously until they reached adult values on day 22.

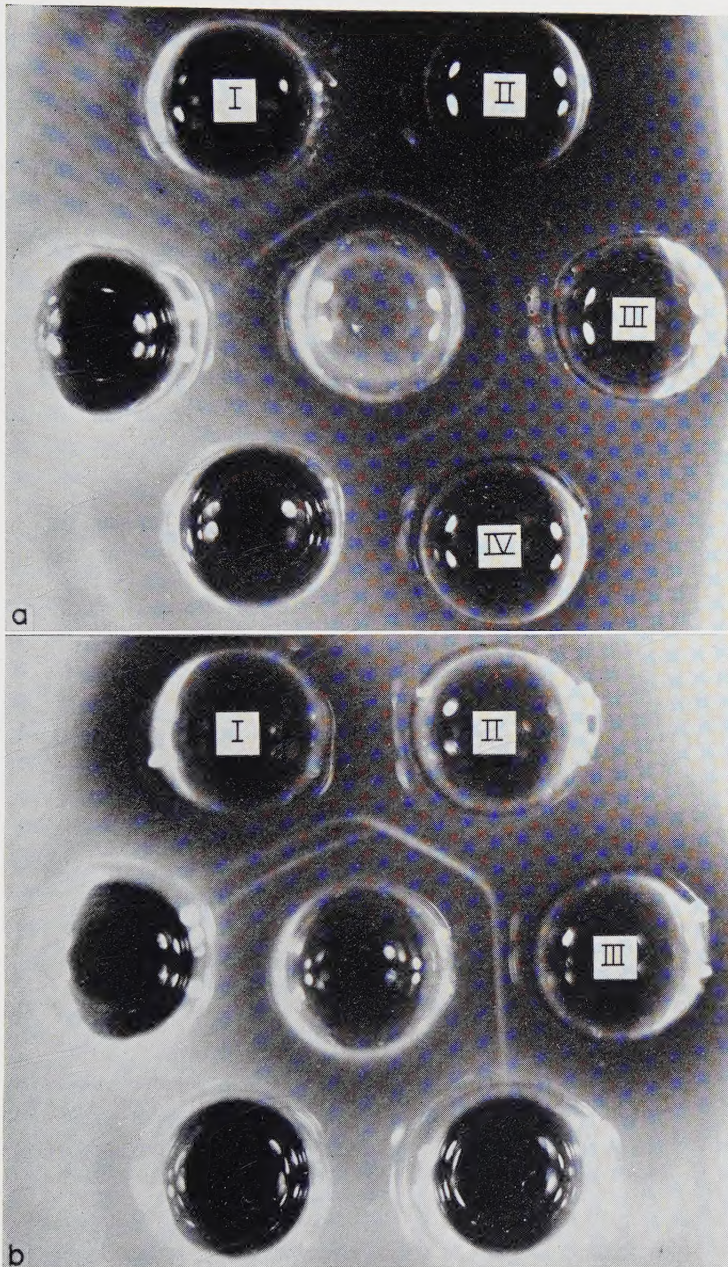


FIG. 2. Double gel diffusion analysis. (a) Anti- μ -chain sera. The central well contained mouse serum taken 4 days after injection of SRBC. (I) Antiserum 26 before absorption and (II) after absorption with IgA, (III) absorbed antiserum 44, (IV) monospecific reference antiserum to IgM from MOPC 104 E tumours. (b) Anti- α -chain serum. The central well contained mouse serum as in (a). (I) Antiserum 1904 before and (II) after absorption with IgM, (III) monospecific reference antiserum to IgA from MOPC 315 tumours.

Chain-specificity of the surface receptors

The anti-IgG serum which had been used for the experiments described above was directed mainly to H-chains, but it also contained weak anti-L-chain activity in double gel diffusion (see Materials and Methods). After absorption with isolated L-chains the cytotoxic activity remained the same with both spleen cells from adults and thymocytes from 5-day-old donors. If however the antiserum was completely absorbed with Cohn fraction II, the cytotoxic reaction was abolished with the cells from both organs (Table 2). The antiserum thus reacted with γ -chain determinants of the receptors in the test system.

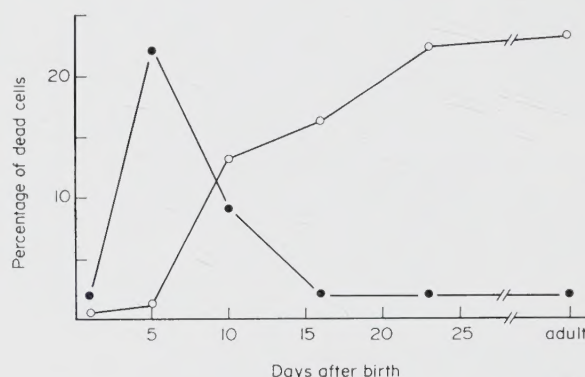


FIG. 3. The postnatal development of γ -chain receptor cells in (●) the thymus and (○) the spleen. Cytotoxic assay with antiserum 2784. The percentage of dead cells represent target cells with γ -chain receptors.

TABLE 2
SPECIFICITY FOR γ -CHAINS OF THE ANTI-IGG SERUM REACTING WITH THYMUS AND SPLEEN CELLS

Anti-IgG serum 2784 diluted 1/10	Percentage dead target cells	
	Spleen, adult	Thymus, 5 days after gestation
Not absorbed	24 (18-28) ‡	23 (20-24) ‡
Absorbed with L-chains *	20, 22	16, 20
Absorbed with Cohn fraction II †	1, 3	3 (1-5) ‡

* One millilitre of antiserum was absorbed with 0.7 mg of isolated L-chains. It was then specific for γ -chains in double gel diffusion.

† One millilitre of antiserum was absorbed with 10 mg of Cohn fraction II. No precipitation line could then be detected in double gel diffusion.

‡ Between three and eight determinations.

Organ specificity of the receptors

Two anti-IgG sera were absorbed with crude membranes of thymocytes, spleen cells or brain tissue from adult animals. They were then assayed with thymocytes from 5-day-old donors and spleen cells from adults. The results are summarized in Table 3. Absorption with brain tissue did not impair the activity of the antisera, and served as a control to exclude non-specific cytotoxic reactions. Absorption with spleen cell membranes abolished the reactivity with spleen cells but the reactivity with thymus cells remained unchanged.

Absorption with thymocyte membranes abolished the reactivity with thymus cells but the reactivity with spleen cells was not altered. Thus thymus-specific and spleen cell-specific γ -chain receptors could be differentiated.

Demonstration of γ -chain receptors on thymus cells by indirect immunofluorescence staining

γ -Receptors could not be revealed on thymocytes from adults in the cytotoxic assay. Nevertheless they were present, possibly within the cells, in a sufficient amount to absorb

TABLE 3

SPECIFIC SURFACE ANTIGENIC DETERMINANTS OF THYMUS AND SPLEEN CELLS WHICH REACTED WITH ANTI-IGG SERA

Anti-IgG serum	*Absorbed with membranes of:	Percentage of dead target cells					
		Thymus, 5th day			Spleen cells, adult		
		1:10 †	1:40	1:160	1:10	1:40	1:160
17	} Thymus, adult	2	0	0	23	15	5
2784		3	0	0	25	16	7
17		21	14	6	1	0	0
2784	} Spleen, adult	20	13	3	0	0	0
17		22	14	5	22	14	4
2784		20	10	3	23	15	10

* One-millilitre aliquots of both antisera were absorbed with one unit amount (12 mg protein) of crude membranes. Absorption with brain did not diminish the cytotoxic activities and served as control.

† Antiserum dilution.

TABLE 4

INDIRECT IMMUNOFLOUORESCENCE STAINING OF LIVING THYMUS CELLS WITH RABBIT ANTI-IGG AND ANTI- γ -CHAIN SERUM

Treatment of anti-IgG serum 2787	Age of thymus donors	Percentage of stained cells *
None	5 days	22, 22, 21
Absorbed with L-chains	5 days	22, 23
Absorbed with L-chains	Adult	None
Absorbed with L-chains and thymus cell membranes from adult donors	5 days	None

* Antiserum 2784 absorbed with Cohn fraction II served as control.

the antisera completely. We wanted to confirm these results by an independent method. The results of the indirect immunofluorescence staining of γ -receptors are represented in Table 4. They gave a good coincidence with the data of the cytotoxic assay. No difference between the relative amount of cells stained with anti-IgG serum and the same serum after absorption with L-chains was observed. Twenty-one to 23 per cent of the cells were stained in thymuses from 5-day-old donors. Also in accord with the results of the cytotoxic assay was the absence of receptors on thymus cells from adults. After absorption with crude membranes of adult thymocytes no fluorescence was detectable on thymocytes from 5-day-old donors.

SURFACE RECEPTORS REACTING WITH ANTI- μ -CHAIN SERUM*The postnatal development of receptor cells in the thymus and the spleen.*

The results of these experiments are summarized in Fig. 4. The relative amount of cells reacting with anti- μ -chain serum in the thymus was 2 per cent at birth. Then it increased in a continuous manner, was 16 per cent on day 5 and reached almost adult values on day 22 (28 per cent). The analogous cells in the spleen appeared 5 days later but the shape of the curve was similar to that of the thymus. On day 5 the relative amount of cells was 2 per cent, increased continuously and reached adult values on day 23 (21 per cent).

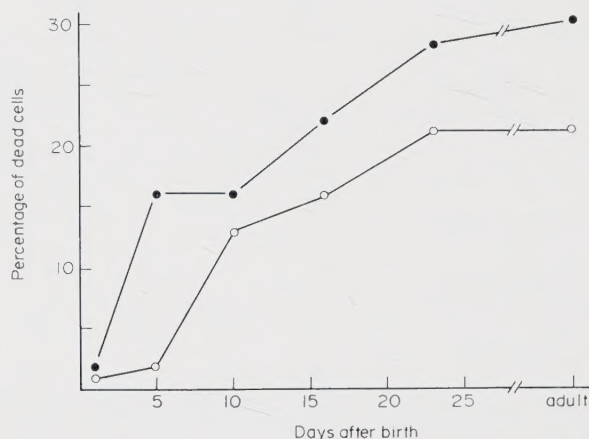


FIG. 4. The postnatal development of μ -chain receptor cells in (●) the thymus and (○) the spleen. Cytotoxic assay with antiserum 26. The percentage of dead cells represent target cells with μ -chain receptors.

TABLE 5

SPECIFICITY AND REPRODUCIBILITY OF THE REACTION OF ANTI- μ CHAIN SERA WITH THYMUS AND SPLEEN CELLS. COMPARISON OF THE REACTIVITY OF ANTI-POLYCLONAL μ CHAIN SERA FROM DIFFERENT SPECIES WITH ANTI-MONOCLONAL μ CHAIN SERUM

Anti- μ -chain serum	Percentage dead target cells					
	Thymus, adult donors			Spleen, adult donors		
	1:10 ‡	1:40	1:160	1:10	1:40	1:160
Rabbit 26 *	21	15	5	30	23	13
Rabbit 26, absorbed with crude IgM	2	0	0	1	0	0
Rabbit 44 *	20	14	6	23	17	9
Guinea-pig 48 *	25	15	8	28	20	10
Guinea-pig 49 *	23	16	7	26	20	12
Rabbit, monoclonal † MOPC 104	4	2	0	25	19	12

* Anti-IgM antisera induced with a partially purified fraction from mouse serum.

† Antiserum induced with IgM protein from MOPC 104 E.

‡ Antiserum dilution.

A comparison of the reactivity of different anti- μ -chain sera

The reactivity of five different anti- μ -chain sera are shown in Table 5. Absorption with partially purified serum IgM abolished the reaction with spleen and thymus cells. The

two rabbit and two guinea-pig antisera to serum IgM (polyclonal IgM) reacted in a very similar manner. They killed 20–25 per cent spleen cells and 23–30 per cent thymus cells from adult donors. The antiserum to IgM from MOPC 104 E tumours (monoclonal IgM) did not react with thymus cells but was able to kill 25 per cent spleen cells.

Organ-specificity of the receptors

The four antisera to polyclonal IgM were absorbed with membranes of brain, spleen or thymus. Their reactivity with thymocytes and spleen cells was then assayed. The results are summarized in Table 6. Absorption with brain did not reduce the activity of the antisera. Absorption with spleen cell membranes abolished the reactivity with spleen cells but not with thymocytes. Absorption with thymus membranes abolished the reactivity with thymus cells but not with spleen cells. These results demonstrated that thymus-specific and spleen-specific μ -chain receptors could be distinguished.

TABLE 6

THYMOCYTE- AND SPLEEN CELL-SPECIFIC ANTIGENIC DETERMINANTS WHICH REACTED WITH ANTI- μ -CHAIN SERUM

Anti- μ -chain serum	Absorbed* with membranes of:	Percentage of dead target cells					
		Thymus, adult			Spleen, adult		
		1:10 †	1:40	1:160	1:10	1:40	1:160
Rabbit 26	Thymus	1	0	0	21	14	5
Rabbit 44		1	0	0	19	14	5
Guinea-pig 48		0	0	0	23	15	6
Guinea-pig 49		1	0	0	23	17	8
Rabbit 26	Spleen	28	20	10	1	0	0
Rabbit 44		22	15	7	2	0	0
Guinea-pig 48		25	17	8	2	0	0
Guinea-pig 49		24	17	9	2	0	0
Rabbit 26	Brain	28	19	7	20	14	6
Rabbit 44		23	16	8	20	14	6
Guinea-pig 48		25	17	9	24	16	8
Guinea-pig 49		23	16	8	23	15	7

* One-millilitre aliquots of the four antisera were absorbed with one unit amount (12 mg protein) of crude membranes. Absorption with brain did not diminish the cytotoxic reaction and served as control.

† Antiserum dilution.

Response of the thymus receptor cells to antigenic stimulation with SRBC

Adult mice were injected i.v. with 5×10^7 SRBC and 4 days later the cells reacting with anti- μ -chain sera were determined in the spleen and the thymus. The results of the experiments are shown in Table 7. The amount of the cells roughly doubled in the thymus to 68 per cent whereas it remained unchanged in the spleen. Again, no cells reacted with anti-monoclonal (MOPC 104 E) μ -chain serum in the thymus of immunized animals. This underlined the thymus specificity of the proliferative response. Further evidence for this was obtained from absorption of the antisera with membranes from normal and immunized animals. Absorption with spleen cell membranes abolished the reactivity of the antisera with spleen cells but not with thymocytes. Absorption of the antisera with thymocyte membranes did not diminish the reactivity with spleen cells, but the reactivity with thymocytes was reduced (to 34 per cent after absorption with normal and to 26 per cent and 21 per cent after absorption with immunized thymocyte membranes). Absorption

TABLE 7

SPECIFIC RESPONSE OF THYMUS CELLS REACTING WITH ANTI- μ -CHAIN SERUM AFTER IMMUNIZATION WITH SRBC*

Anti- μ -chain serum	Absorbed† with membranes of:	Percentage of dead target cells of immunized animals					
		Thymus			Spleen		
		1:10 ‡	1:640	1:10240	1:10	1:40	1:160
26	—	67	38	5	22	15	6
44	—	68	43	4	21	15	8
26	Spleen, normal	65	31	4	1	0	0
44		66	12	1	2	1	0
26	Spleen, immunized *	67	26	4	1	—	—
44		66	40	1	2	—	—
26	Thymus, normal	34	14	2	21	14	6
44		34	12	1	25	18	10
26	Thymus, immunized *	26	11	2	20	14	6
44		21	10	2	24	17	9
26	Brain, normal	66	—	—	21	—	—
44		67	—	—	23	—	—
26	Brain, immunized *	67	18	2	20	14	5
44		65	—	—	21	—	—
26 absorbed with IgM		1	—	—	—	—	—
Rabbit anti-MOPC 104		4	0	0	25	19	12

* The animals were immunized intravenously with 5×10^7 SRBC and killed 4 days later.

† One-millilitre aliquots of the antisera were absorbed with one unit amount (= 12 mg protein) of membranes.

‡ Antiserum dilution.

with brain membranes did not reduce the reactivity of the antisera but absorption with serum IgM abolished it. The partial reduction of reactivity after absorption with thymus membranes was further investigated by sequential absorptions of the antisera with unit amounts (see Materials and Methods section) of thymus membranes. The results of these experiments are shown in Table 8. The activity of the antiserum with homologous cells was abolished after four absorptions with membranes from immunized animals. In contrast absorption with membranes from normal thymocytes did not further diminish activity with

TABLE 8

CONSECUTIVE ABSORPTION OF ANTI- μ -CHAIN SERUM WITH THYMOCYTE MEMBRANES OF IMMUNIZED AND NORMAL ANIMALS. EVIDENCE FOR THE EXPOSURE OF NEW μ -CHAIN ANTIGENIC DETERMINANTS ON THYMOCYTES AFTER IMMUNIZATION WITH SRBC

Anti- μ -chain serum, rabbit 26		Percentage of dead target thymus cells of immunized animals		
		1:10	1:160	1:640
Not absorbed		67	52	38
Absorbed with thymocyte membranes of immunized animals	* 1 ×	26	17	12
	2 ×	18	10	5
	3 ×	12	3	0
	† 4 ×	4	0	0
Absorbed with thymocyte membranes of normal animals	* 1 ×	34	24	4
	2 ×	33	22	13
	3 ×	33	20	10

* Two aliquots of the antiserum were consecutively absorbed with unit amounts (= 12 mg protein per 1.0 ml) thymocyte membranes of immunized and normal animals.

† The antiserum sample which had been absorbed four times with membranes of immunized animals was still able to kill 21 per cent of spleen cells at a 1:10 dilution (compare with Table 7).

thymus cells from immunized animals. The reactivity with spleen cells remained unaltered after the four absorptions with thymus membranes. These results suggested that new, class-specific receptor determinants appeared on thymocytes after short-time immunization with SRBC.

Localization of γ - and μ -chain receptors on different cell populations

Equal parts of anti- γ - and anti- μ -chain serum were mixed and assayed with thymocytes from 5-day-old donors and with spleen cells from adults. The mixed antisera killed approximately the same numbers of cells (40 per cent thymocytes, 38 per cent spleen cells) as the sum of those killed by each antiserum alone (thymocytes: 16 per cent with anti- γ ; 22 per cent with anti- μ . Spleen cells: 20 per cent with anti- γ ; 21 per cent with anti- μ). This strongly suggested that the receptors of both classes were present on different cell populations. However a small number of double producers would not have been detected by our method.

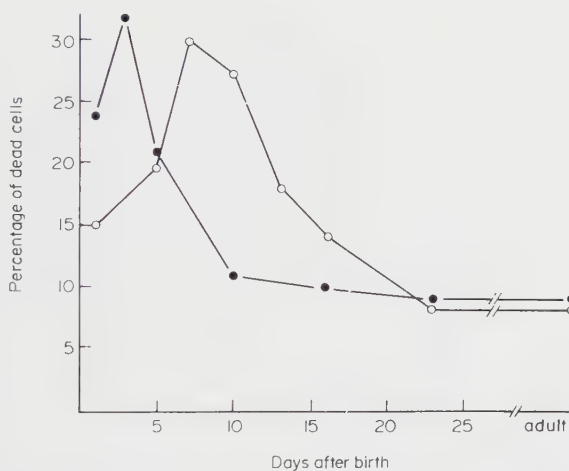


FIG. 5. The postnatal development of α -chain receptor cells in (●) the thymus and (○) the spleen. Cytotoxic assay with antiserum 1904. The percentage of dead cells represent target cells with α -chain receptors.

SURFACE RECEPTORS REACTING WITH ANTI- α -CHAIN SERUM

The postnatal development of α -receptor cells

The results of these experiments are represented in Fig. 5. Relatively high numbers of α -receptor cells were already present at birth in the thymus (24 per cent) and the spleen (15 per cent). During the following days they increased further and reached a maximal value on day 3 in the thymus (32 per cent) and on day 7 in the spleen (30 per cent). The relative numbers then decreased and reached the values of the adult organs (8–10 per cent) on day 23.

Response of α -receptor cells to antigenic stimulation and the organ specificity of the receptors

Adult mice were immunized with SRBC as already described. The α -receptor cells responded to antigenic stimulation qualitatively in the same way as the μ -receptor cells (Table 9). The relative amount increased to 25 per cent in the thymus, whereas the spleen

cells remained unchanged. The reactivity was abolished by absorption of the antiserum with IgA. The organ specificity of the receptors was then investigated and the results of these experiments are also shown in Table 9. The anti- α -serum was absorbed with membranes of brain, thymus or spleen from immunized animals. Absorption with brain did not diminish the cytotoxic activity with spleen and thymus cells. Absorption with spleen abolished the activity of the antiserum with spleen cells, but the reactivity with thymus cells remained unchanged. Absorption of the antiserum with thymus abolished the reactivity with normal thymus cells but reduced it only from 25 per cent to 15 per cent with thymus cells from immunized animals. Three further successive absorptions abolished the reaction with immunized thymus cells. The reactivity of the thymus-absorbed antiserum with spleen cells remained unchanged. These results suggested that thymus-specific and spleen cell-specific α -receptors could be distinguished. They also showed that new, thymus-specific α -determinants appeared after short-time immunization with SRBC.

TABLE 9

THYMUS AND SPLEEN CELLS REACTING WITH ANTI- α -CHAIN SERUM. SPLEEN- AND THYMUS-SPECIFIC ANTIGENIC DETERMINANTS. APPEARANCE OF NEW ANTIGENIC DETERMINANTS AFTER IMMUNIZATION WITH SRBC

Anti- α -chain serum rabbit 1904	Percentage of dead target cells							
	Thymus				Spleen			
	Normal		Immunized		Normal		Immunized	
	1:10 ‡	1:40	1:10	1:40	1:10	1:40	1:10	1:40
Not absorbed	9	5	25	19	8	5	9	4
Absorbed with brain from immunized animals *	9	4	24	19	8	5	8	5
Absorbed with spleen from immunized animals *	9	5	24	18	0	0	1	0
Absorbed with thymus from immunized animals †	1 ×	1	0	15	12	8	6	10
	2 ×			10	7			
	3 ×			7	5			
	4 ×			1	0			
Absorbed with thymus from normal animals	1	0	17	11	9	4	9	6

* Aliquots of antiserum were absorbed with one unit amount (= 12 mg protein per 1.0 ml serum) of membranes

† An aliquot of antiserum was absorbed in consecutive steps with unit amounts (= 12 mg protein/1.0 ml antiserum) of membranes.

‡ Antiserum dilution.

Presence of α - and μ -chain receptors on different cell populations

Equal amounts of anti- α and anti- μ -chain serum were mixed and assayed with thymus cells of immunized animals. The mixed antisera were able to kill 86 per cent thymus cells, which was the additive total for both antisera acting alone. This suggested that the α - and μ -receptors were present on different cell populations in the immunized thymus.

ONTOGENESIS OF PLAQUE-FORMING CELLS (PFC)

Different strains of mice differ with respect to their immunological maturity at birth and during the first weeks of life (Playfair, 1968). Results with our strain of mice were thus

compared with the reported results of others with respect to the ontogenesis of PFC. 10^8 SRBC were injected i.p. and 4 days later the number of direct plaques was assayed (Playfair, 1968). The thymus did not contain PFC confirming the results of others (Evans, Williamson and Irvine, 1968). PFC were not detectable in the spleen until day 7 after birth, but on day 10 significant amounts were present ($144/10^6$ cells). Then they increased rapidly and on day 12 roughly half the amount ($826/10^6$ cells) of adult values ($1473/10^6$ cells) was reached. Our strain of mice was thus as immature immunologically as the C 57/Bl 6 (Playfair, 1968) and the Charles River mice (Pierpaoli, Fabris and Sorkin, 1971) with respect to PFC.

STRAIN SPECIFICITY OF THE RECEPTOR CELLS

The relative amount of μ - and α -receptor cells did not differ significantly between Swiss mice and the C57/Bl 6 strain in the thymus and the spleen.

DISCUSSION

Our results strongly suggested that thymus cells of the mouse contained surface receptors of the IgM, IgA and IgG immunoglobulin classes.

The ontogenetic development of receptor cells was characteristic and distinct for each of the three immunoglobulin classes in the thymus and the spleen. The transient appearance of high relative amounts of γ -receptor cells in the thymus and of α -receptor cells in the thymus and the spleen during the first week of life may reflect distinct states of differentiation of these immune organs. No significant amounts of multiple H-chain producers were detected by our method and thus we have to assume that the class-specific cell pools expanded and regressed independently. Two general statements can however be made. First, the α -cell pools preceded the appearance of the μ - and the γ -cells in both the thymus and the spleen. These were the only receptor cells already present at birth. Second, the receptor cells of all three classes in the thymus preceded the appearance of the analogous cells in the spleen by 3–5 days. Since the thymus cell receptors of all three classes were not related immunochemically to the receptors of the spleen we cannot assume that the receptor cells in the thymus were B cells migrating through this organ.

The expansion and regression of a distinct cell pool in the young thymus has been observed previously (Fellenberg *et al.*, 1972). A specific surface membrane antigen, having a B-cell distribution in the adult, developed in the thymus of the neonate according to the same time course as the γ -receptor cells. So far we have no direct evidence that both antigens are located on the same cell population. Since the antigen has not yet been named we propose to call it ' γ -like' or GL antigen.

The biological function of large, transient receptor cell pools during the first week of life remains obscure. At this time no humoral Ig's are yet secreted (reviewed by Solomon, 1971). On the other hand the period during which tolerance can be induced to the strong H2 histocompatibility antigens is restricted to the first week of life (Billingham and Brent, 1959). Induction of tolerance to soluble antigens in the neonate has shown that IgG-producing cells are more susceptible than IgM-secreting cells (Nossal and Austin, 1966; Williams, 1966). An interesting *in vitro* correlation is the capacity of thymocytes to produce a mixed lymphocyte interaction during the first week of life which disappears between the tenth and the twentieth day (Howe, Goldstein and Battisto, 1970; Goldstein, Guha, Howe and White, 1971). Self recognition has been postulated as the biological explanation of this

phenomenon (Howe *et al.*, 1970) and we also have been tempted to speculate that the transient γ - and α -receptor cells could be involved in self tolerance.

Absorption of the class-specific antisera with membranes of thymocytes and spleen cells showed that the receptors of the thymocytes were not related immunochemically to the receptors of spleen cells. This underlines the well-known difference between thymus cells and lymphoid cells of peripheral lymphoid organs. Antigenic determinants of both spleen and thymus specificity however were present in the serum, since the activity of the antisera with thymus and spleen cells could be completely absorbed with serum immunoglobulins. One question which remains to be solved is whether thymus immunoglobulins are distinct populations of circulating antibodies with specific functions.

The selective response of α - and μ -receptor cells in the adult thymus to short-time immunization with SRBC clearly demonstrated that they are involved in the immune response. The reaction consisted in the proliferation of thymus cells that contained new, class-specific antigenic determinants. It is conceivable that they resembled 'individual antigenic determinants' (Hopper and Nisonoff, 1971) which were membrane-bound; the rabbit antisera which were used to detect them had been induced with mouse immunoglobulin containing antibodies to SRBC (see Materials and Methods section). This proposal remains tentative since we did not establish directly the specificity of the new receptors for SRBC. Recently however membrane-bound idiotypes have been detected on human blood lymphocytes by cytotoxic assay (Wernet *et al.*, 1972).

The biological function of the reactive thymocytes with their receptors remains obscure. We have nevertheless tried to formulate a possible function based on the theory which described bridging of B and T cells by antigen as a possible step in cell cooperation for humoral antibody formation (Mitchison *et al.*, 1970; Raff, 1973). Our proposal is that thymus cell receptors could compete with either T or B cells for the bridging antigen and by this mechanism inhibit and control the production of humoral 'aggressive' antibodies. Complete inhibition would be manifested as tolerance. An analogous competitive mechanism could also be conceived for cell-mediated immune reactions and the previously postulated self recognition process in neonatal mice.

The presence of immunoglobulin receptors on thymus cells has remained a controversial and frustrating issue among immunologists. Since the initial experiments by Raff (1970) it has become almost a dogma for many investigators that thymocytes and T-cells do not contain surface immunoglobulins. Some reports however have described thymus IgM as a 7S molecule after solubilization (Marchalonis *et al.*, 1972; Cone *et al.*, 1972). A comparison of the reactivity of all our anti- μ -chain sera with thymocytes and spleen cells suggests a possible reason for this controversy. All antisera which had been induced with IgM of normal, SRBC-stimulated mice reacted with thymus cells. The only antiserum which failed to be cytotoxic for thymocytes had been induced with IgM myeloma protein from MOPC 104 E tumours. This antiserum reacted however with spleen cells to the same extent as the others. We tentatively conclude from this difference that the monoclonal IgM from the widely used plasma cell line may lack the antigenic determinants present on thymocyte receptors. The data in the literature give some support to this idea. Investigators who could reveal IgM on thymus cells used antisera to normal IgM (Nossal *et al.*, 1972, Marchalonis *et al.*, 1972) whereas others who failed to demonstrate it used antisera to MOPC 104 E protein (Rabellino *et al.*, 1971; Vitetta *et al.*, 1971, 1972; Lamelin *et al.*, 1972).

A doubt remains whether our chain-specific antisera were in fact contaminated. It is

conceivable that they all reproducibly contained antibodies to antigens not detectable by gel precipitation methods. However, a comparison of the relative numbers of spleen cells killed by our class-specific antisera in the cytotoxic assay with the values published previously using different methods (Raff, 1970; Rabellino *et al.*, 1971; Lamelin *et al.*, 1972) gives fairly good concurrence. It suggests that the cytotoxic antibodies were in fact directed against H-chains only. A second piece of evidence, which we consider more convincing, is the fact that the surface molecular structures detected on thymocytes reacted to antigenic stimulation. What is more tempting than to consider these molecules as antibodies?

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